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New chimera proteins for fluorescence correlation spectroscopy

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A new class of chimera proteins has been developed. They are ideally suited for detection by fluorescence correlation spectroscopy (FCS), a new technology to analyze molecular interactions. The molecular structure of these chimera proteins consists of four domains: a N-terminal $(His)_6$-tag for affinity chromatography followed by an eight amino acid epitope for immunodetection, a polypeptide affinity domain (ADF) for target specific interaction and a C-terminal Green Fluorescent Protein (GFPuv) for reporting of interaction with the target by FCS. We designed, prepared and characterized a prototype of ADF-GFP proteins capable of specific interaction with DNA fragments bearing nuclear factor (NF)-κB sites. ADF NF-κB p50 and a non-DNA-binding deletion mutant (p35) combined with GFPuv were inserted in a procaryotic vector and expressed in *E. coli*. Following affinity purification the fluoroproteins p50-GFPuv and p35-GFPuv were employed in specific protein-protein and protein-DNA interaction studies. FCS analysis as well as EMSA showed that p50-GFPuv revealed a fully functional ADF. We present a model for the preparation of GFP fusion proteins capable of specific interaction with proteins, lipids or nucleic acids. The rational design allows any polypeptide fragment to be incorporated into the chimeric protein. So a new series of bio-molecules with different binding specificities and assays can be developed.

1. Introduction

Fluorescence correlation spectroscopy (FCS) is a novel method to analyse molecular interactions in liquid environment (Rigler 1995; Korn et al. 2003). In contrast to conventional fluorescence spectroscopy, FCS uses an extremely small volume element for the determination of the emitted light. By the reduced light scattering a high signal-to-noise ratio due to the confocal imaging is obtained, which dramatically increases the sensitivity. In principle, FCS employs a focused laser beam for excitation of a fluorophore in a 10^{-15} l volume. Single molecules diffusing through the illuminated volume element give rise to bursts of fluorescence light quanta. A single-photon detection unit records each individual burst resulting from a single molecule over time. At concentrations >1 nM, diffusion events of more than one particle are registered as fluctuations of the emitted fluorescence intensity. Autocorrelation of the recorded fluorescence fluctuations reveals the number of molecules in the illuminated volume element, as well as their characteristic transitional diffusion time. Since diffusion of a molecule depends on the molecular weight and shape, complex formation with a larger binding partner increases the diffusion time of the fluorescent aggregate. This principle allows discrimination between the bound and free states of the fluorophore.

To study molecular complex formation new fluorescent probes are required, which can specifically recognize and interact with any target molecule of interest. Since the diffusion time of a particle is proportional to the third root of the molecular weight, an at least 5-fold increase of the analyte's molecular mass by binding to its target molecule is required for reliable discrimination of free and bound state of the analyte in FCS. Fluorescence labelled antibodies are endowed with the required specificity for interaction, but their molecular weights often exceed those or are close (∼140 kDa) to the size of the macromolecular subjects to be analysed, such as proteins, lipids and nucleic acids. Fluorescence labelled natural ligands of receptors (Schuler et al. 1999), screened combinatorial peptides (∼5 kDa) libraries (Cwirla et al. 1990) or single chain antibodies (∼30 kDa) selected against the molecular target of interest may fulfill the demands of FCS-analysis (Schwalbach et al. 2000). However, engineering of these labelled proteins is usually time-consuming and additionally they often do not provide a high affinity ($<10^{-5}$) to their target. Currently, no convenient protocol is available for the rapid production and characterization of fluorescent reagents appropriate for FCS studies.

To address these problems we started to develop a group of novel analytes, based on small affin-domain fragments (ADF), provided by recent molecular cloning studies (Trier et al. 1999). Appropriately chosen ADFs with naturally high avidity and specificity to the target can be genetically fused to the Green Fluorescent Protein (GFPuv), which provides an instant fluorophore capability of the analyte. In addition to high affinity of the fusion protein, the ADF-GFPuv chimera can report complex formation

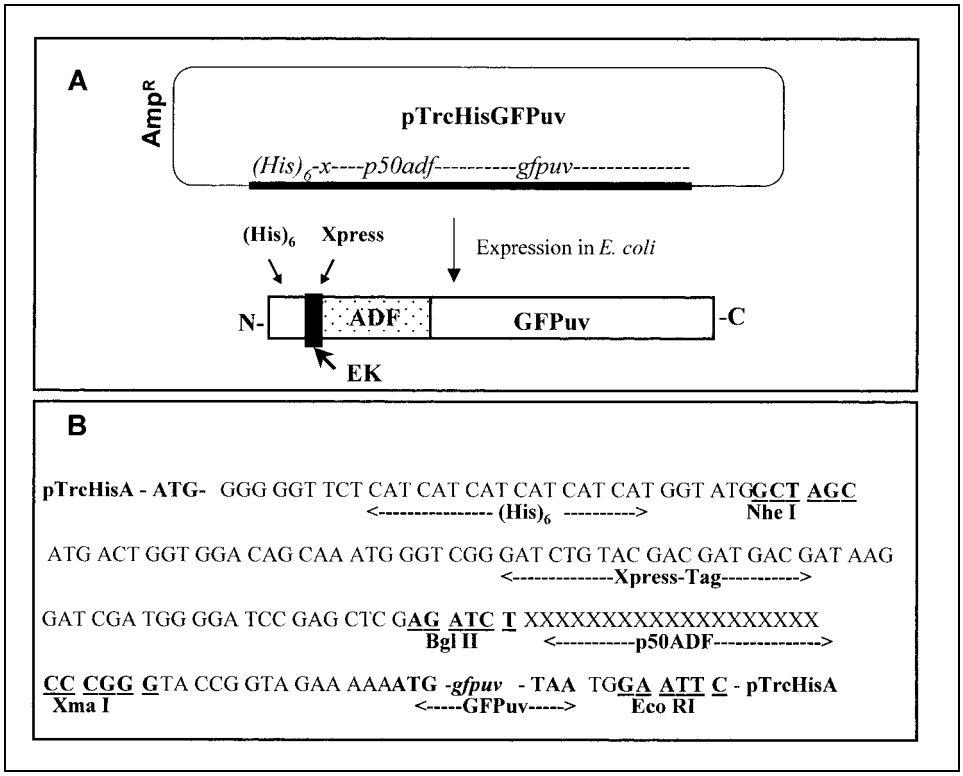

Fig. 1:
Schematic diagram in panel A demonstrates the structure of the pTrcHisGFPuv vector coding for the p50-GFPuv fusion protein. The ATG start codon followed by a $(His)_6$ repeat and the Xpress epitope, and the enterokinase cleavage site (EK), were provided by the parental pTrcHisA vector. The *gfpuv* gene was subcloned from the pGFPuv plasmid with the Pst I and Eco RI restriction enzyme sites. Panel B shows a detailed DNA-structure of the pTrcHisGFPuv. The pTrcHisA bacterial expression plasmid was inserted with the p50, as an affin domain fragment (ADF). To obtain different target specificity any other ADF of interest can be inserted at the Bg II-Xma I sites into the plasmid (B)

with macromolecules due to green fluorescence, which might be ideal in FCS studies. As a prototype of the ADF-GFPuv chimera proteins the DNA binding motif of nuclear factor-κB p50 (NF-κB p50) was fused to GFP and experiments were designed to characterize the p50-GFPuv fusion protein.

Currently, a number of structurally related proteins of the NF-κB family has been recognized, among which p50 and p65 (also known as RelA) are the best known (Ganchi et al. 1993). In each member of the NF-κB family the Rel-homology domain (RHD) is preserved, required for dimerization and specific DNA binding. As a prototype of the NF-κB family serves p50 first identified as a transcription factor binding to a defined DNA sequence primarily localized in the κ immunoglobulin light-chain enhancer region of B-cells. Later it has been recognized that κB sites exist in many other promotors and expression of NF-κB p50 is not confined to the immune system (Kaltschmidt et al. 1994). NF-κB p50 (~50 kDa) is synthesized as a ~110 kDa precursor molecule (Costello et al. 1993; Watanabe et al. 1993). Unlike other transcription factors, p110 is anchored in the cytosol to inhibitory proteins, termed IκBs, which keep the NF-κB complexes in an inactive state. Stimulation with various inducers (e.g. cytokines, mitogenes and pathogens) results in a rapid degradation of IκB proteins and liberated NF-κB p50 translocates to the nucleus (Hansen et al. 1992). Upon heterodimerisation with p65 or homodimerisation the complex binds to κB sites present in numerous promoters and activates expression of protein products involved in inflammation, immune response, apoptosis and cell proliferation (Ishikawa et al. 1993; Baeuerle and Henkel 1994; Muller et al. 1995; Ghosh et al. 1998).

Here we designed, prepared and characterized a prototype of the ADF-GFPuv chimera proteins, capable of specific interaction with DNA fragments bearing κB sites. FCS experiments, carried out with the p50-GFPuv analyte and the κB containing large size DNA (~500 kDa), proved complex formation in solution. The p50, genetically fused to the GFPuv fluorophore, served as a potent reporter of interaction determined by FCS. Our experiments with the p50-GFPuv chimera protein demonstrated that ADF-GFP fusion proteins can be produced rapidly and are well suitable for macromolecular analysis employing FCS.

2. Investigations and results

2.1. Preparation and purification of p50- and p35-GFPuv

Recently we have demonstrated that the GFPuv is a useful fluorophore for FCS investigations (Trier et al. 1999). To extend the use of this reagent, a convenient tagging vector, the pTrcHisGFPuv plasmid was prepared (Fig. 1A), which can be inserted with any ADF of interest (Fig. 1B). A remarkable feature of the pTrcHisGFPuv vector is that it can express any recombinant protein with an N-terminal $(His)_6$-repeat followed by an epitope of eight amino acids. The His-tag serves for metal affinity purification whereas the epitope allows characterization of the purified protein by the anti-Xpress antibody. Any ADF to be expressed obtains N-terminal affinity and detection tags while at the C-terminal the GFPuv can serve as fluorescence marker. The anti-express antibody epitope also incorporates a enterokinase cleavage site, which allows elimination of these tags after purification and immunocharacterization.

To demonstrate the usefulness of this vector we expressed the p50-GFPuv chimera protein and then studied the interaction of p50-GFPuv with its corresponding DNA-binding site (Fig. 1A). As a control we constructed a second chimeric protein with a partially deleted Rel domain, termed p35-GFPuv, which is theoretically unable to bind to the κB site. Both fusion proteins, p50- and p35-GFPuv, were expressed in *E. coli*. To recover the recombinant proteins, crude homogenate of bacteria was applied on Ni-affinity column whereby the N-terminal $(His)_6$ tag could interact with the Ni immobilized on the column resin.

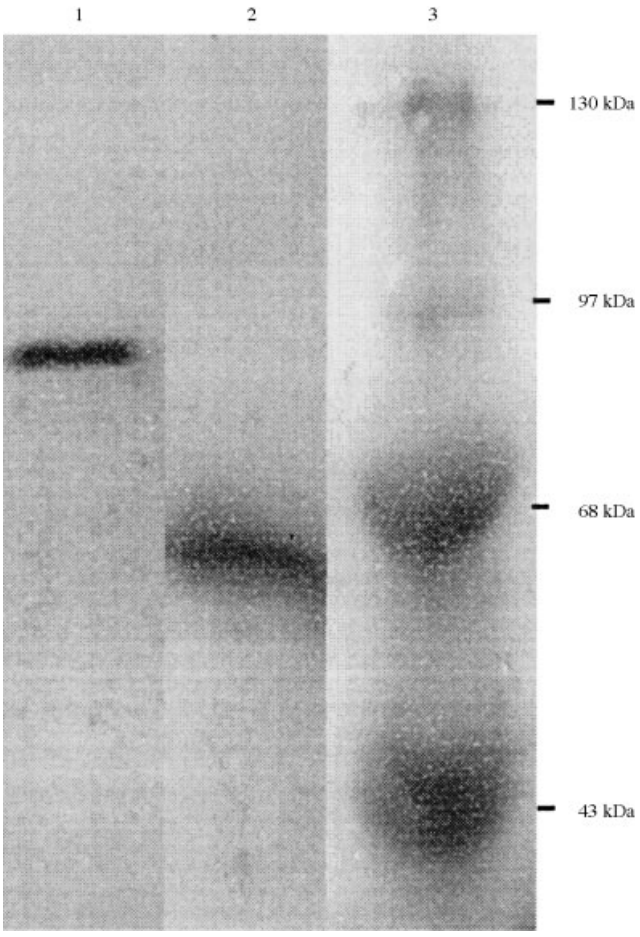

Fig. 2: Immunoblot of the recombinant proteins. Western blot analysis of p50-GFPuv (lane 1) and p35-GFP (lane 2) with the anti-Xpress antibody were carried out as described in the Experimental section. Molecular weight markers (lane 3)

2.2. Characterization of p50- and p35-GFPuv chimeras

For further characterization, the affinity purified proteins were subjected to Western blot analysis. Detection with the anti-Xpress antibody specific for the N-terminal epitope resulted in major bands at ~80 (p50-GFPuv) and ~65 kDa (p35-GFPuv) further confirming the expected structure of the recombinant proteins (Fig. 2). These results suggest the correct expression of both p50- and p35-GFPuv.

To prove the fluorescence properties of the purified p50-GFPuv required for FCS detection we examined the recombinant transcription factor with the STORM scanner's blue laser excitation line (450 nm). The fluorescence of the GFPuv, linked to p50, was preserved even after SDS-PAGE separation. Indeed transillumination demonstrated high fluorescence in the crude extract and in the eluted fractions (Fig. 3). Purification by Ni^{2+}-affinity chromatography led to the main protein bands at ~80 kDa (p50-GFPuv), ~65 kDa (p35-GFPuv) and ~30 kDa (GFPuv, $(His)_6$ tagged), which is in good agreement with the expected molecular weight of GFPuv and the recombinant proteins. Two independent purification experiments, carried out with crude bacterial extracts, elucidated the reproducibility of the Ni^{2+}-affinity chromatography.

To substantiate that the spectral properties of the fluoroproteins are essentially unaltered we compared the fluorimetric spectra of the fusion proteins and GFPuv. Fig. 4 clearly shows that both recombinant proteins contain fully functional fluorophore activity. Blue light was maximal absorbed at 395 nm and green light was emitted at 509 nm which is identical to the non-fused GFPuv.

In the following FCS served for further characterization of the recombinant proteins. Fluctuations in the fluorescence intensity were recorded while p50-, and p35-GFPuv were diffusing through the confocal volume element. Fluorescence and low FCS detection limits allow both fluoroproteins to be examined at concentrations of 2–5 nM. Among the limiting factors of FCS detection photobleaching and the amount of photons residing in the triplet state are the most important variables. The triplet value represents the percentage of molecules that pass from the excited singulet energy level to a triplet state before they go back to the ground energy level without emitting fluorescence light. As molecules on the triplet level do not contribute to the fluoresence signal, fluorescent dyes with a high triplet level are less suitable for FCS analysis. FCS revealed a good resistance for photobleaching determined with p50- and p35-GFPuv. More than 90% of the emitted fluorescence was preserved after 60 min of illumination. Moreover the triplet amount was less than 15%. These values are similar to that observed with free GFPuv (data not shown). It should be mentioned that conventional fluores-

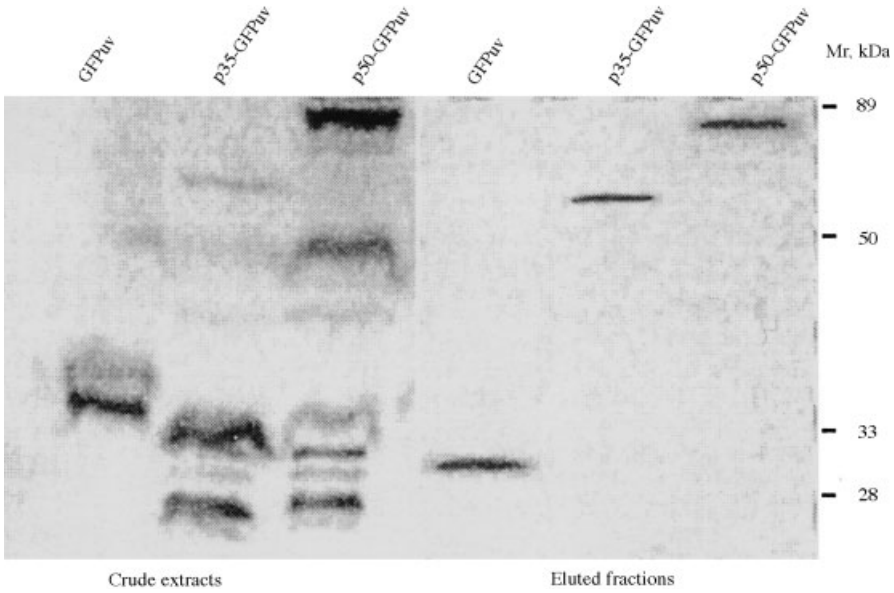

Fig. 3:
SDS gel electrophoresis and UV laser scanning florimetry of crude extract and eluted fractions of Ni-affinity purification of GFPuv, p35- and p50-GFPuv. Samples separated without boiling in denaturing gel were subjected to florimetry in the STORM fluorimeter. In the eluted fractions the protein bands at ~30, ~65, and 80 kDa represent the $(His)_6$ tagged GFPuv, the partially Rel domain deleated p35-, and the active p50-GFPuv recombinant proteins, respectively

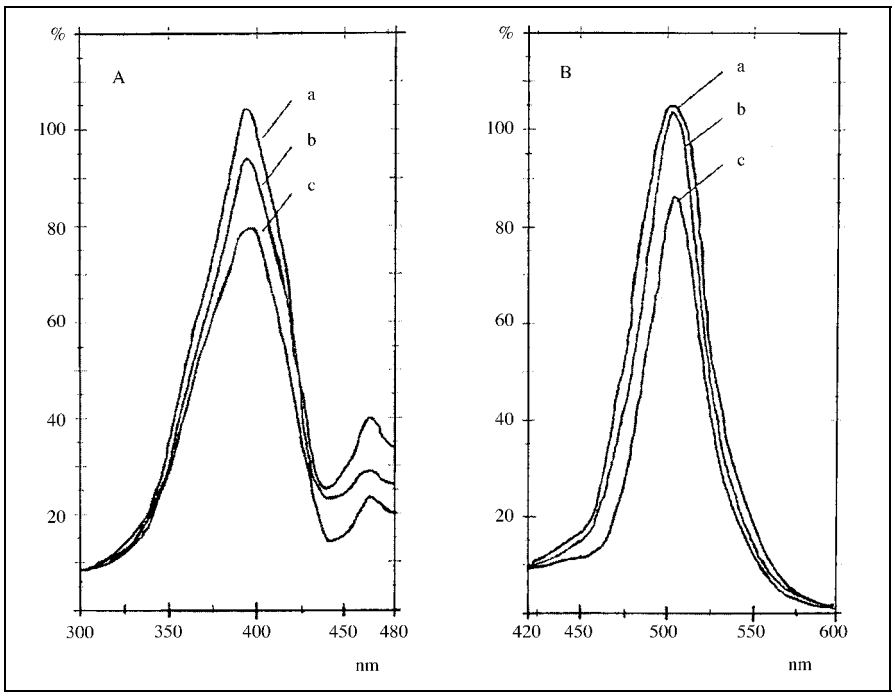

Fig. 4:
Absorption (A) and emission (B) spectra of p50-GFP (a), p35-GFP (b) and GFPuv (c). Excitation of the fluoroproteins leads to the characteristic maximum at 395 nm, whereas the emission maximum for all three proteins is 509 nm. So fusion to p50 or p35 does not change the spectral maxima of GFP

cence markers are characterized by considerably higher values for both triplet amount and photodestruction.
We next studied the diffusion time of the fusion proteins by FCS. The diffusion time of p50-GFPuv was 400 µs, whereas p35-GFPuv showed a lower diffusion value of 345 µs. Diffusion times are well in accordance with their molecular weights of 80 and 65 kDa, respectively.

2.3. Protein- and DNA interaction of p50- and p35-GFPuv chimeras

First, the DNA-binding capacity of p50-GFPuv was studied with conventional gel shift assays. Interaction of p50-GFPuv with its κB DNA binding site was determined by EMSA. The DNA fragment coding for a κB element was labelled with ^{32}P, then the radioactive oligo probe was mixed with p50- and p35-GFPuv. A typical EMSA is presented in Fig. 5, which clearly demonstrates that p50-GFPuv induced a large shift in native gel, whereas the control protein did not affect the mobility of the γ-^{32}P-DNA. Addition of an anti-p50-antibody resulted in a supershift of p50-GFPuv, indicating that there is a specific binding of the recombinant transcription factor to its corresponding DNA binding fragment (Fig. 5).
Moreover it was of interest whether FCS technology is a useful tool to monitor molecular interactions of the recombinant proteins. First a specific protein-protein interaction was studied using the anti-Xpress-antibody. In agreement with the supershift determined by EMSA, the diffusion time of p50-GFPuv increased from 398 to 473 µs confirming the formation of a protein-protein complex between the p50-GFPuv and the anti-Xpress-antibody specific for the detection tag incorporated in the pTrcHisGFP vector. This association was highly specific, as other antibodies did not influence the diffusion time of p50-GFPuv (data not shown).
In the following the interaction of the p50-GFPuv with its corresponding DNA binding site was studied. A reporter plasmid was used, in which tandem κB sites were incorporated. Addition of the κB reporter plasmid to p50-GFPuv resulted in a remarkable change in the autocorrela-

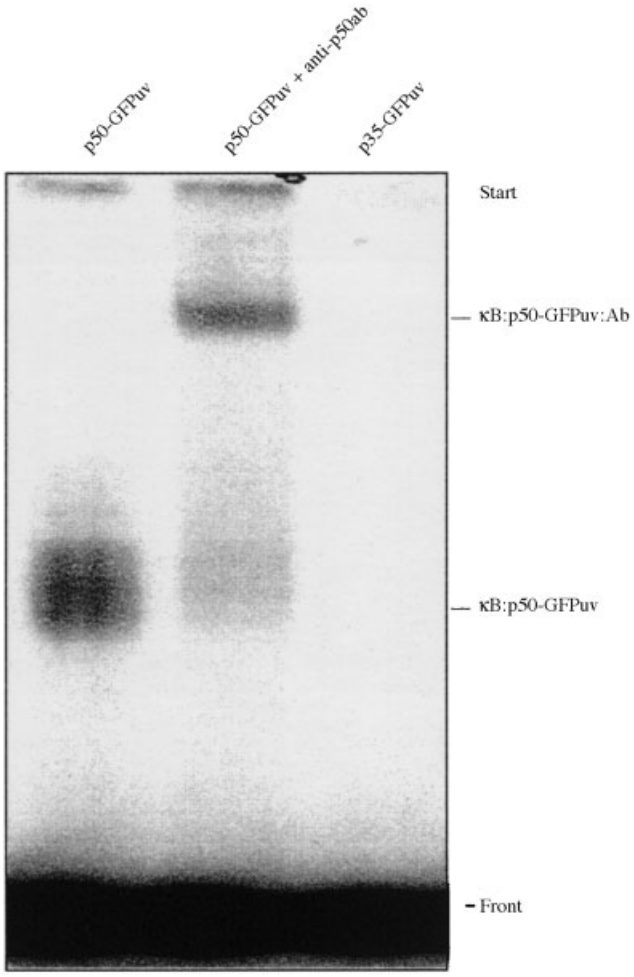

Fig. 5: Electrophoretic Mobility Shift Assay with p50- and p35-GFPuv. ^{32}P labeled κB DNA was mixed with the affinity purified p50-GFPuv and EMSA was carried out as described in the Experimental section. Interaction between the oligo DNA and the recombinant protein resulted in a slowly migrating κB: p50-GFPuv complex (lane 1). Addition of an anti-p50 specific antibody to this complex induced a supersift of κB: p50-GFPuv:Ab complex (lane 2). p35-GFPuv was unable to bind the κB oligo DNA (lane 3)

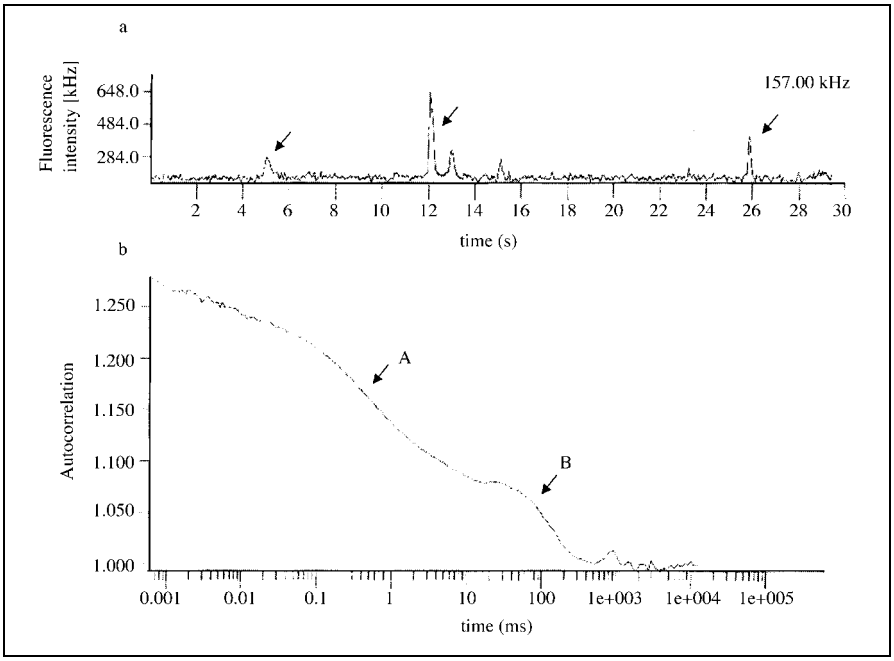

Fig. 6:
FCS fluorescence intensity (a) and autocorrelation function (b) of p50-GFP following addition of its corresponding κB binding site. Autocorrelation curve indicates that the recombinant transcription factor has bound to the reporter plasmid: A represents diffusion of free p50-GFP and B corresponds to the slower diffusing complex of p50-GFP and DNA. Shift of curve B to the right towards extremely high diffusion times and big fluorescence intensity peaks – marked by arrows – indicate that several complexes have formed to large molecular aggregates

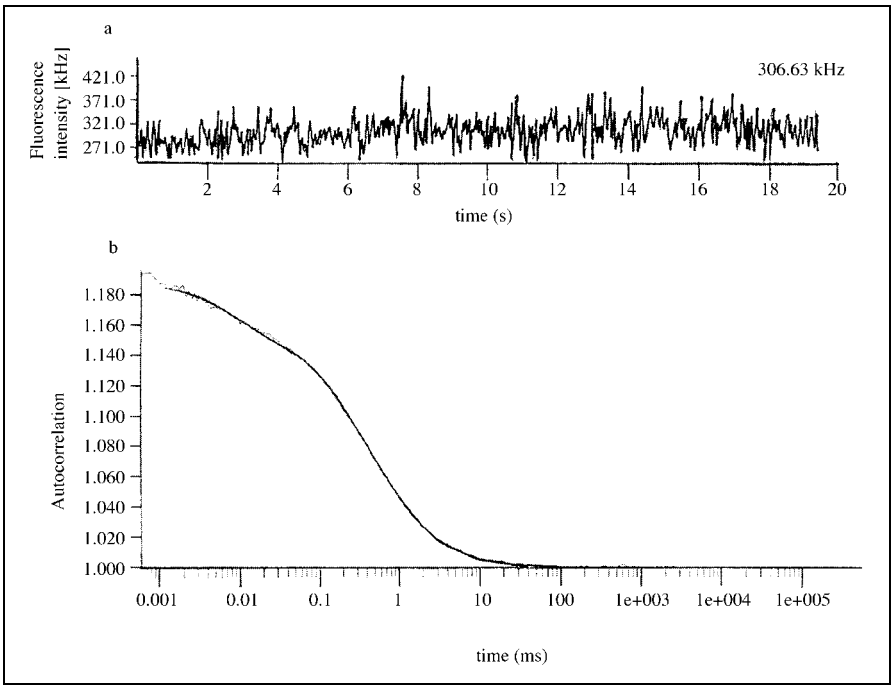

Fig. 7:
FCS fluorescence intensity (a) and autocorrelation function (b) of p35-GFPuv. Addition of the p50 reporter plasmid does neither influence autocorrelation function of p35-GFPuv nor fluorescence intensity course. Mean diffusion time of 345 µs does not change and large fluorescence intensity peaks are not detectable. Therefore the control protein does not bind to DNA indicating specific DNA-binding of p50-GFP

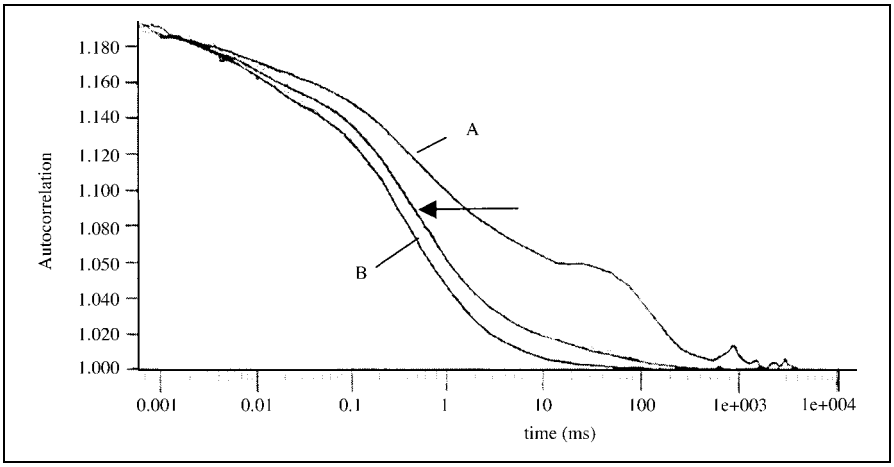

Fig. 8:
FCS autocorrelation function of p50-GFPuv bound to its reporter plasmid (A) following addition of native p50. p50 in tenfold excess causes a shift of the correlation curve to the left (see arrow) towards the diffusion time of free p50-GFP (B). This implies that p50-GFP was completely displaced by the native transcription factor

tion curve of p50-GFP. Fig. 6b shows two levels in the calculated function, A representing the diffusion of free p50-GFPuv while B corresponds to the considerably slower diffusing complex of DNA: p50-GFPuv. It should be mentioned that by binding to the reporter plasmid the molecular mass of the recombinant transcription factor is drastically increased to 3900 kDa. In contrast to p50-GFPuv, the correlation function of the control p35-GFPuv was not affected by addition of the plasmid (Fig. 7b), demonstrating that p50-GFPuv specifically bound to the κB binding sites.

Moreover, high peaks were detected in the fluorescence intensity curve of p50-GFPuv during FCS measurement (Fig. 6a). These peaks, which did not appear in the intensity curve of the control protein (Fig. 7a), are characteristic for large molecular associates. Additionally the shift of the autocorrelation curve (b) towards enormously high diffusion times implies that a variety of NF-κB-DNA complexes must have been formed.

To further verify the specificity of the DNA-binding, competition experiments with a tenfold excess of native p50 were conducted. Upon addition of p50 to the complex diffusion time decreased until autocorrelation function revealed a diffusion time of 398 μs, which is equivalent to that of free p50-GFPuv (Fig. 8). Simultaneously, the large peaks indicative of molecular aggregates disappeared. So the p50-GFPuv fusion protein was completely displaced by native p50.

3. Discussion

The study of specific molecular interactions has become increasingly important in molecular biological research. FCS is ideally suited for this purpose, because it enables molecular interactions to be observed on-line in homogeneous assays. The high sensitivity of the FCS technology combined with small sample volumes and short measurement times warrants a broad range of applications (Maiti et al. 1997). As FCS technology is based on the detection of fluorescence light the analyte has to be labelled with a fluorophore. Many conventional applications employ fluorescence marker dyes, which, however, often reveal a high triplet fraction. For example, with fluorescein up to 30% of the emitted light is lost due to transition into triplet state. Moreover photobleaching is responsible for another reduction by 50%. In contrast, the novel GFPuv fluorophore possesses much better efficiency. FCS experiments of GFPuv alone and as part of a fusion protein proved a low triplet value as well as very good photostability (Chalfie et al. 1994).

Due to its superior fluorescence properties GFP was used for the creation of chimera proteins produced by the novel pTrcHisGFPuv vector. The pTrcHisGFPuv vector is endowed with internal multicloning sites for rapid production of recombinant analytes prepared with any ADF of interest. Here as ADF NF-κB *p50* and *p35*, coding for a non-DNA-binding deletion mutant, were incorporated into the vector. A repeat of six histidine molecules, integrated at the N-terminus of the fusion protein, allows instant purification by Ni^{2+}-affinity chromatography. In addition to the rapid purification, high affinity binding to the Ni^{2+}-chelate affinity column further ensures a correct reading frame of the synthesized polypeptide. An additional benefit of the $(His)_6$-tag is that it defines the proper translation initiation of the recombinant protein. The eight amino acid epitope after the $(His)_6$-repeat provides immunodetection by the anti-Xpress antibody. The incorporated enterokinase cleavage site allows sequence specific proteolytic elimination of the majority of the N-terminal extensions. In our studies the biological function of p50-GFPuv was not affected by these tags. Characterization of the recombinant protein can be futher improved due to the fluorescence capability of the C-terminal GFPuv, which is even easily detectable in SDS-PAGE. Here UV illumination was used to monitor the purification steps. The molecular weight of the chimeric proteins was determined by laser scanning fluorimetry after separation in SDS-PAGE and by Western blot. We present evidence that GFPuv as an integral part of the fluoroprotein reveals excitation and emission spectra similar to GFPuv alone. This finding gives the most compelling evidence that GFPuv is an inert and efficient fluorescence reporter.

EMSA and FCS analysis of the p50-GFPuv verified that neither N-, nor C-terminal tagging influences the known biological activity of the NF-κB p50 domain fragment. As it was demonstrated by protein-protein and protein-DNA binding studies ADF-GFPuv chimeras can extend the potential of fluorescent technologies in molecular diagnosis, including the relatively new FCS. In FCS, specific binding of both the p50-, and p35-GFPuv to the anti-Xpress antibody were detected by increased diffusion time as compared to the free analyte, indicative of complex formation with the target. Furthermore, FCS-analysis, as well as EMSA clearly showed that merely the p50-GFPuv can bind to the κB DNA. The Rel domain truncated p35-GFPuv neither can produce gel shift nor increase the diffusion time, again indicative of specific interaction of p50-GFPuv with the κB sites.

Taken together, we experimented here with prototypes of GFPuv fusion proteins. The model protein with a fully functional ADF, such as the p50 domain was capable of unique interaction with macromolecules, either proteins or nucleic acids. A chimera prepared with a truncated ADF, p35-GFPuv served as negative control. p50-GFPuv demonstrated that it is ideal for use in FCS detection. Particles ≥ 140 kDa performed well in FCS, as verified by epitope-antibody interaction, but even much higher protein-DNA complexes were also emanable for detection. FCS technology appears feasible with a variety of polypeptide fragments incorporated in the pTrcHisGFPuv vector.

In many cases mutations inducing a single amino acid substitution in the primary structure of a protein can result in severe diseases like cystic fibrosis or even autonomous cell growth (Bos 1989). To develop substances for mutagenesis detection (e.g. highly specific single-chain antibodies) the mutated structure can be integrated as affinity domain in the GFP fusion protein. The fluoroprotein can then be used as a tool to select produced single chain antibodies specific for the mutated structure. Interactions between the recombinant single chain antibody and the mutated domain can then be followed on-line by FCS.

Moreover GFP fusion proteins seem ideally suited for FCS screening studies. Indeed some years ago Rigler described the potential of this technology for screening a large number of molecules (Rigler 1995). Recently, Auer et al. demonstrated that FCS allows detection of e.g. topoisomerase inhibitors in high-throughput-screening studies (Auer et al. 1998). With its short assay times – only seconds per well – and a low detection limit in the nanomolar range FCS appears as an excellent screening tool. Fusion of the target structure with GFP providing high resistance to photobleaching allows interactions with a vast number of molecules to be monitored by FCS.

We present a model for the preparation of GFP fusion proteins capable of unique interaction with proteins, lipids or nucleic acids ideally suited for FCS detection. The rational design allows an entirely new series of bio-molecules and assays – relevant for novel pharmacological and diagnostic agents – to be developed.

4. Experimental

4.1. Materials

Cell culture products were obtained from Life Technologies (Eggenstein, Germany). Phenylmethylsulfonylfluoride (PMSF), protease inhibitors, such as leupeptin, pepstatin A, aprotinin and other general chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany).

4.2. Plasmid vectors and recombinant proteins

Prokaryotic vectors coding for the GFPuv, pTrcHisA, and pNF-kB-Luc were purchased from Clontech (Palo Alto, CA), Invitrogen (San Diego, CA), and Stratagene, (La Jolla, CA), respectively. Recombinant rGFPuv, purified to homogeneity, was from Clontech (Heidelberg, Germany).

4.3. Genetic engineering of the pTrcHisGFPuv tagging vector

Generally applied recombinant techniques were used, similar to those described earlier (Olah et al. 1994; Trier et al. 1999). Briefly, to prepare the pTrcHisGFPuv vector the pTrcHisA plasmid (Invitrogen) was cut at the Pst I and Eco RI and a DNA fragment coding for the GFPuv was subcloned with the same sites derived from the pGFPuv vector (Clontech) (Fig. 1). The finished vector backbone can be easily modified to express any other ADF-GFPuv of interest in bacteria by inserting the PCR-amplified cDNA, coding for the appropriate ADF in the multi cloning site of the pTrcHisGFPuv plasmid (Fig 1, panel B).

4.4. Assembly of chimeric genes coding for p50- and p35-GFPuv

The p50-GFPuv chimera gene was assembled in the pTrcHisGFPuv vector. Human NF-κB p50 gene-specific oligo DNAs, mutated to **Bgl II** restriction enzyme sites (CTC G**AG ATC T**GC ATG GCA GAA GAT GAT CCA TAT TTG and CTC G**AG ATC T**AT GG ACC CAA GGA CAT GGT GGT CGG C) served as the forward primers to amplify the p50, and the p35 cDNA, coding for the N-terminal DNA-binding, Rel-homology domain fragment (1–418 amino acids), and a non-binding deletion mutant (92–418 amino acids), respectively. For both of the fragments, a reverse primer ACC GGT A**CC CGG G**GA GTA ATC CCA CCA TAA GTA GGA AAT CC with **Xma I** site was used. To obtain the cDNA fragments a plasmid, containing the coding for the 1–418 amino acid residues of the human p50, was used in the PCR. Following digestion with Bgl II and Xma I enzymes, the fragments were gel-purified and inserted into the Eco RI – Xma I sites of the pTrcHisGFPuv vector.

4.5. Affinity purification of p50- and p35-GFPuv

p35- and p50-GFPuv fusion proteins were expressed in E. coli and purified according to the following procedure. To an overnight culture of the cells in LB-medium containing 50 μg/ml ampicillin 0.4 mM IPTG (Calbiochem, Bad Soden, Germany) was added. Following 4 h of shaking at 37 °C cells were collected by centrifugation at 10000 × g for 10 min. The following steps were performed at 4 °C. The supernatant was decanted and the bacterial pellet was subsequently resuspended in ice-cold Novagen binding buffer (Boehringer Ingelheim Bioproducts Partnership, Heidelberg, Germany) containing 1 mM PMSF, leupeptin, aprotinin and pepstatin A (each 20 μg/ml). Cells were sonicated on ice to shear DNA, separated from cell debris by centrifugation at 10000 × g for 10 min. Extraction was repeated. Combined supernatants were centrifuged at 3900 × g for 20 min and used for affinity purification according to the manufacturers protocol (Novagen His Bind Buffer Kit). The His-tag sequence of the recombinant proteins bound to Ni^{2+}, which was immobilised on a His bind metal chelat resin. After unbound proteins were washed away the fusion proteins were recovered by elution with 1 M imidazole. The fluorescence of the eluted fraction was controlled by FCS.

4.6. Western blot analysis

The expression of the fusion proteins was studied using enhanced chemoluminiscence (ECL) Western blot analysis. Affinity purified fluoroproteins were separated in a 10% SDS-PAGE. Prestained proteins from Calbiochem (Bad Soden, Germany) served as molecular weight markers for electrophoresis combined with biotinylated molecular weight standards from New England Biolabs (Schwalbach, Germany) for the transfer to PVDF membrane (Millipore, Eschborn, Germany). Following electrophoresis the gels were immersed in transfer buffer (Tris-glycine, pH 8.3) for 15 min. Proteins were transferred to PVDF membrane using a Biometra (Göttingen, Germany) tank blot (12 h, 100 mA). The membranes were blocked with 5% non-fat dried milk in TBST for 1 h under permanent shaking. Before the addition of primary antibody membranes were washed three times for 5 min with TBST. Then the membranes were incubated with the anti-Xpress antibody (Invitrogen, NV Leek, The Netherlands) diluted 1 : 5000 in TBST and 1% BSA. After three washing steps a 1 : 1000 diluted solution of horseradish peroxidase (HRP) linked antiserum (New England Biolabs) was added for 1 h under permanent shaking. HRP linked anti-biotin antibody (New England Biolabs) served for detection of biotinylated molecular weight standards. Non-bound antibody was removed by three washing steps and membranes were incubated with LumiGlo detection solution (New England Biolabs). The light emitted by destabilised LumiGlo reagent was subsequently captured on X-ray film. All incubation and washing steps were performed at room temperature.

4.7. Electrophoretic Mobility Shift Assay (EMSA)

NF-κB consensus sequence, double-stranded-GTCAGTCA**GGGGAATTCC**CA TCGGTCAG, was end-labelled with [γ-P^{32}] ATP (ICN, CA) using T4 polynucleotide kinase according to the manufacturers instructions (New England Biolabs). The labelled oligonucleotide was separated from free [γ-^{32}P]ATP using a G-50 Sephadex spin column (5′-Prime 3′-Prime, Inc., Boulder, CO). Protein extracts (10 μg) were evaluated for NF-κB DNA-binding activity by incubation with labelled oligonucleotide in a buffer containing 10 mM Tris-HCl, pH = 8.0, 40 mM NaCl, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol, 5% glycerol and 3 μg poly-(dI-dC) (Pharmacia Biotech Inc.) for 30 min at room temperature. Specificity of the binding was defined by competition with excess unlabelled consensus oligonucleotide, no competition was obtained with a mutant oligonucleotide. The specificity also was checked with a supershift assay. For the supershift assay 150 ng of anti-p50 antibody or as a control an antibody unable to interact with the NF-κB p50 (Santa Cruz Biotechnology, CA) was added. After 20 min of incubation at 20 °C samples were separated in 9% native, tris/glycine polyacrylamide gel and the NF-κB complexes were visualized by autoradiography.

4.8. Fluorescence Correlation Spectroscopy (FCS)

FCS was performed with a ConfoCor (Zeiss, Jena/ Evotec, Hamburg, Germany) using a C-Apochromat 40 x/1.2 W korr objective. The confocal volume element was illuminated by an $argon^{+}$-laser at the 488 nm line. An optical density filter OD 1.0 results in optimal illumination output. Fluorescence of p50-GFPuv and p35-GFPuv (2–5 nM) was recorded at room temperature in eight chamber coverglasses (Nunc GmbH, Wiesbaden) for 20–30 s. To study anti-Xpress-antibody interactions 5 nM fluoroproteins were incubated with a 100-fold excess of antibody for 15 min. For examination of DNA-interactions 20 nM of p50-GFP or p35-GFP were incubated with the reporter plasmid in a fivefold excess. In competition experiments a tenfold excess of native p50 (Promega, Mannheim) was added. The samples were diluted with Novagen binding buffer. Data evaluation was done with the FCS ACCESS software package (Zeiss/Evotec) using the one-component fit model.

4.9. Fluorimetry

Fluorimetric measurements of p50-/p35-GFPuv and GFPuv were conducted with a Kontron SFM 25 fluorimeter (Kontron Instruments, Milan, Italy) at concentrations between 0.1 and 1 μM. The determination was carried out in the buffer which was used for binding studies.

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